20 A Homogeneous, Fluorescent Polarization Assay for Inositol 1,4,5-Trisphosphate (Ins P_3)

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CONTENTS

20.1 INTRODUCTION

G-protein–coupled receptors (GPCRs) are one of the largest classes of drug discovery targets [1,2]. GPCR ligands regulate cellular and physiological pathways by signaling through several second messengers, including cyclic AMP, inositol phospholipids, and calcium [3]. Quantitation of second messengers is frequently used as a means to screen and pharmacologically characterize GPCR ligands [4]. The GPCR signaling process occurs by two major pathways. GPCRs coupling to $G\alpha_s$ and $G\alpha_i$ proteins activate or inhibit, respectively, adenylate cyclase and subsequently change intracellular cAMP levels. GPCRs coupling to $G\alpha_q$ or $G\alpha_o$ proteins activate phosphoinositol phospholipase Cβ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) forming *sn* 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (Ins P_3) [5]. Ins P_3 binds and opens an endoplasmic Ins P_3 gated calcium channel, causing release of bound calcium into the cytosol [6]. Several metabolic products of Ins P_3 also modulate cellular function, including inositol 1,3,4,5-P_4 (Ins P_4), which acts to facilitate Ins P_3-mediated calcium release synergistically [7].

There are several HTS assay systems to measure intracellular cyclic AMP as a marker of G_s- and G_i-coupled GPCRs [8]. In contrast, there are few assays available to selectively measure Ins P_3 to monitor Gq-coupled GPCR activation, particularly those suitable for automated HTS. Consequently, many HTS laboratories measure changes in intracellular calcium to assay G_q-coupled GPCRs using a fluorescent calcium-sensitive dye, loaded into intact cells as a cell-permeable ester. Real-time changes in the GPCR-induced signal are then determined in a microtiter plate using imaging instruments, such as a fluorescent imaging plate reader system (FLIPR, Molecular Devices Corp) [9].

Exhibit "1"

Screening library compounds, however, may modulate intracellular calcium levels by other means than binding to the receptor, such as nonspecific blockade of calcium channels or exacerbated intracellular calcium release. Moreover, compounds that autofluoresce or quench fluorescence result in ambiguous changes in the assay signal and may manifest as false-positive or -negative hits. Consequently, several assays have been developed to measure GPCR-induced inositide phospholipid hydrolysis [10–12]. The majority of these assays involve radioactive measurements, many of which are suboptimal for high-volume screening.

20.2 MEASURING INOSITOL PHOSPHOLIPID HYDROLYSIS TO MONITOR GPCR ACTIVATION

A proportion of GPCRs that couple to $G\alpha_q$ proteins activate phospholipase C and mobilize Ins P_3 [5–7]. Measurement of GPCR-induced changes in phosphoinositide phospholipase C activity is frequently undertaken by measuring inositol phosphate production. Here, tritiated inositol is incorporated into the inositol phospholipids of the cell. Activation of the receptor results in release of radiolabeled Ins P_3. The experiments are conducted in the presence of lithium, which inhibits inositol monophosphate phosphatase, thereby blocking the cycle and increasing accumulation of the tritiated isotope at the monophosphate form. This radiometric approach is used in conjunction with scintillation proximity assay (SPA) technology (GE Healthcare) to provide a homogeneous platform more suitable for automation [13].

20.3 MEASURING INOSITOL PHOSPHATE LEVELS TO MONITOR GPCR RESPONSES

The measurement of the second messenger, Ins P_3 *specifically*, is undertaken differently and has traditionally been done using mass assays with gas liquid chromatography (GLC), anion exchange chromatography, or high performance liquid chromatography (HPLC) [14]. These techniques, while very sensitive, are not adaptable to assays requiring high throughput. The recognition that Ins P_3 binds to a specific intracellular receptor provides the basis for a radiometric competition binding assay [5]. Here, tritium-labeled Ins P_3 is displaced from a crude preparation of the Ins P_3 receptor using a competition radioligand binding protocol [15,16]. A commercial version of this radioreceptor assay is available from GE Healthcare using bovine adrenal gland Ins P_3 receptor preparations. This format, again when used with SPA, is high throughput [6]. However, the economics of isotopic waste disposal emanating from high-volume screens remains a significant issue.

A nonisotopic assay for Ins P_3 is now available based on the AlphaScreen technology (PerkinElmer). This technique is an amplified luminescence assay that employs donor and acceptor beads. When the donor bead is excited with light at 680 nm, a photosensitizer converts O_2 to singlet oxygen. When two beads are in close proximity, the singlet oxygen produces a chemiluminescent signal in the acceptor bead, activating bead fluorophores and amplifying the signal. In an Ins P_3 assay, the two beads are held in close proximity by a biotinylated Ins P_3 molecule, as the donor bead is coated with streptavidin and the acceptor bead is coated with an Ins P_3-binding protein. In the absence of cell stimulation, a signal is seen. In the presence of free Ins P_3 from the cell, the donor and acceptor beads dissociate, and the signal proportionally decreases [17]. Echelon Biosciences have utilized the AlphaScreen assay format using a binding protein that binds a range of inositol phosphates, including IP_2 and IP_4. These cellular metabolites compete with a biotinylated inositol phosphate analog as described above [18]. This assay has an advantage in that it detects several phosphoinositols, although an extensive evaluation in HTS screens has not been reported to date. Despite the advantage of the AlphaScreen approach as a nonisotopic homogeneous technology, the signal is sensitive to compound quenching, and ambient fluctuations in light and temperature need to be carefully controlled [19]. The AlphaScreen Ins P_3 assay is also lim-

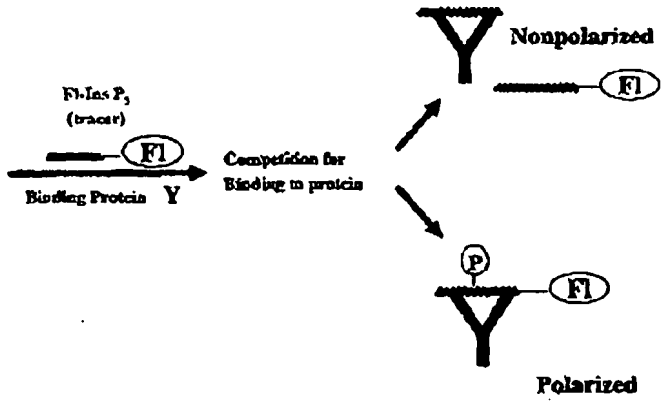


FIGURE 20.1 Schematic representation of the Ins P_3 FP assay principle.

the number of cells per well, as matrix interferences from cell lysates reduce the signal. This issue, plus the instability of the Ins P_3 binding protein preparation, may cause variability in the assay performance and sensitivity.

20.4 HITHUNTER FLUORESCENCE POLARIZATION (FP) ASSAY FOR INOSITOL 1,4,5-TRISPHOSPHATE (INS P_3)

The HitHunter FP Ins P_3 assay from DiscoveRx is a competitive binding assay, in which cellular Ins P_3 displaces a fluorescent derivative of Ins P_3 from a specific binding protein. The assay measures changes in fluorescence polarization (FP), a single-wavelength ratiometric technique, in which a fluorescent derivative of Ins P_3 is used as a tracer. FP is determined as a ratio of fluorescence emissions in the vertical and horizontal planes. When fluorescent molecules are excited with polarized light, the degree to which the emitted light retains polarization reflects the rotation that the molecule underwent between excitation and emission. Small molecules rotate rapidly, and emitted light is random with respect to the plane of emission. When bound to a large protein (such as receptor or antibody), the molecule rotates much more slowly and the emitted light retains more of its polarization. This is measured as an increase in the FP signal.

When excited with polarized light, the emission from a fluorescent derivative of Ins P_3 (tracer) is depolarized compared to the exciting light, due to the rapid rotation of the molecule between excitation and emission. When the Ins P_3 derivative binds to a binding protein, the rotation time is reduced and a high polarization value is seen. In the assay unlabeled Ins P_3, either a standard Ins P_3 solution or derived from the cell lysate, displaces the tracer from the binding protein, and the rotation time increases and low FP signal is measured (Figure 20.1). By this means a calibration is generated to the standard Ins P_3 dilutions, and the molar concentration of Ins P_3 in the cell lysate determined by interpolation (Figure 20.2).

The critical components of the DiscoveRx assay are thus the fluorescent Ins P_3 tracer and the Ins P_3 binding protein, as shown in the protocol in Figure 20.3. In the case of the tracer, three dye conjugates have been developed including a green (fluorescein) derivative of Ins P_3 (Figure 20.4). As low concentrations of fluorescent tracers are used in the assay, the technique is sensitive to optical interference from screening library compounds. The ratiometric processing of the data corrects to some extent for fluorescent compounds. Artifacts or interferences can also be identified by measuring compound fluorescence in the absence of the Ins P_3 tracer. For this reason, the Ins P_3 assay has also been developed for a series of "red" tracers that are less prone to compound

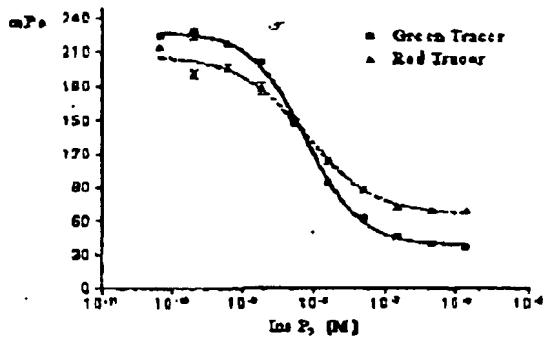


FIGURE 20.2 Ins P_3 standard curve. A standard curve was generated to measure levels of exogenously added Ins P_3. A high concentration of Ins P_3 at 7 μM was serially diluted 1:3 in Ins P_3 standard dilution buffer. Different concentrations of Ins P_3 were incubated with PCA, followed by the addition of the tracer and then the Ins P_3 binding protein. The reaction was read on a multiwell fluorescence polarization plate reader such as the Beckman-Coulter CRI Affinity or LJL Analyst. The majority of the experimental data for this publication was collected on a Beckman-Coulter CRI Affinity, unless noted. An IC_{50} of ~7 to 9 nM was observed when using either the green or red Ins P_3 fluorescent tracers.

TABLE 20.1
Assay Precision of Ins P_3 FP Assay Using Different Tracers

	Green Tracer	Red Tracer
mPs Low standard	238	196
mPs High standard	37	70
S/B ratio/μmPs	6/201	3/126
EC_{50}, nM Ins P_3	9	7
Average % CV of replicates	2	2
Z' Factor	0.97	0.92

Note: n = 4 replicates.

interference (Table 20.1). In all cases the sensitivity of the assay is similar, although changes in FP (denoted as the delta mP) vary according to the dye in question (Figure 20.5 and Table 20.2).

The FP Ins P_3 assay is performed in crude cell lysates, thereby avoiding laborious separation and filtration steps. It is therefore important that the Ins P_3 binding protein exhibit high affinity and selectivity for the D-*myo*-1,4,5-inositol-Ins P_3 isomer over other inositol polyphosphates. The buffer (20 m*M* HEPES, 150 m*M* NaCl, 1 m*M* DTT, 0.1% BGG, and 0.02% Tween 20, pH 7.5) used in the Ins P_3 assay is optimized to ensure high-affinity binding, and competition binding studies with several substituted inositol phosphates demonstrate that the Ins P_3 binding protein is specific for the D-myo-1,4,5 inositol Ins P_3 isomer (Table 20.3). In terms of stability, the performance did not change after 6 h at room temperature (Figure 20.6a) and can withstand multiple freeze/thaw cycles when stored at –80°C. The binding protein is also a stable reagent for more than 2 months at –80°C (Figure 20.6b).

In a similar fashion to many FP-based assays, the DiscoveRx Ins P_3 FP assay is amenable to assay automation systems. A representative standard curve dispensed by a BioMek 2000 liquid handler instrument is shown in Figure 20.7. Here, standard concentrations of Ins P_3 were run in replicates of 10. A coefficient of variance of 2% and a Z' factor of 0.92 to 0.97 are generally achieved. Similar assay performances have been observed using either an Analyst FP reader or a CRI Infinity reader (Figure 20.8).

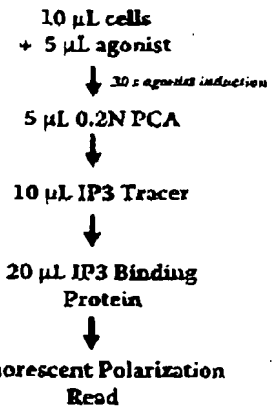


FIGURE 20.3 RiboHunter Ins P_3FP assay protocol. Schematic representation of the steps and additions made to measure levels of Ins P_3.

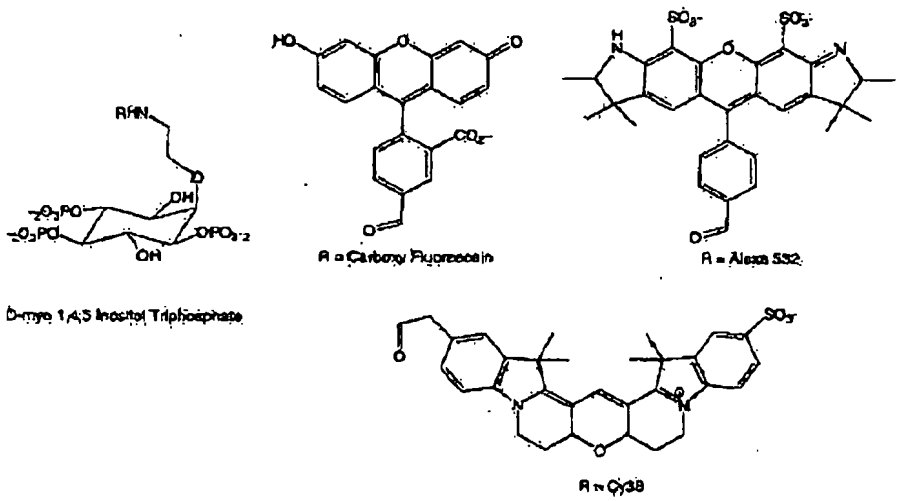


FIGURE 20.4 Chemical structure of the Ins P_3 FP tracer. Amine derivatized D-*myo*-1,4,5-inositoltriphosphoric acid was reacted to each of the hydroxysuccinimide activated carboxy fluorescein, AlexaFluor, and Cy3B dyes separately in dry dimethyl formamide. Each of the Ins P_3 tracers was purified to 99.9% homogeneity by reverse phase HPLC on C18 column and triethyl ammonium acetate: acetonitrile gradient. The molecular weight of all the conjugates was corroborated by electrospray mass spectroscopy.

20.5 MEASURING GPCR AGONISM AND ANTAGONISM

In a similar fashion to other second messengers such as adenylate cyclase, basal and stimulated levels of Ins P_3 are highly dependent on cell number. To correlate cell number with Ins P_3 basal levels, three different CHO-M1 cell lines were studied using the green Ins P_3 tracer. As the cell number per well was increased from 5000 to 50,000, the basal levels of Ins P_3 increased in proportion (Figure 20.9A). These data indicate that the assay is applicable to a range of different cell densities.

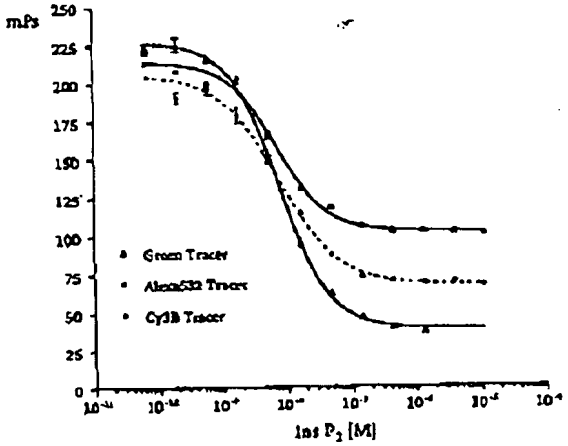
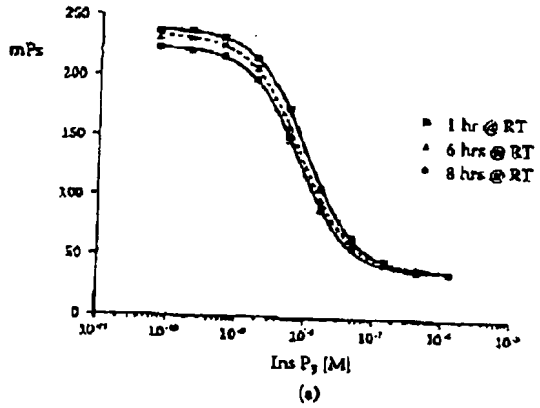
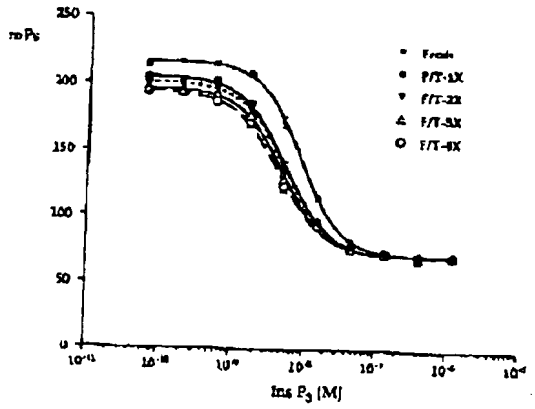


FIGURE 20.5 Alternative red-shifted dye tracers used for the Ins P_3 FP assay. To address issues of compound library interferences by autofluorescence or quenching that may occur using a fluorescein-based Ins P_3 tracer, two different red-shifted Ins P_3 tracers were synthesized and tested. Standard curves using the three different



(a)



(b)

FIGURE 20.6 Stability of the Ins P_3 binding protein. The reagents were equilibrated at room temperature for 1, 6, and 8 h. After each time period, a standard curve titrating Ins P_3 was run. As shown in panel (a), the binding protein was stable over 8 h at room temperature. The sensitivity of the standard curve over 1, 6, and 8 h was 10, 8, and 9 n*M*, respectively. In panel (b), the Ins P_3 binding protein reagent was subjected to four freeze/thaw cycles (–80°C to room temperature) and a standard curve was run. Freshly prepared Ins P_3 binding protein (closed square) IC_{50} = 9 n*M*, (closed circle) one freeze/thaw IC_{50} = 5 n*M*, (closed inverted triangle) two freeze/thaws IC_{50} = 5 n*M*, (open triangle) three freeze/thaws IC_{50} = 5 n*M*, (open circle) four freeze/thaws IC_{50} = 4 n*M*.

Experience has also shown that several different types of cell (CHO-K1, HEK 293 cells, and so on) can be used in the assay (Figure 20.9B).

The goal of a competitive Ins P_3 assay is to measure changes in cellular Ins P_3 concentration induced by GPCR agonist activation. It is well known that the cellular metabolism of Ins P_3 is extremely rapid; after an initial spike, the levels decline to a plateau, the height of which depends upon the cell type and perhaps cytosolic calcium concentration. In some cells, Ins P_3 peak levels oscillate in a frequency that directly correlates to the calcium oscillation frequency [20]. In an "end-point" assay for Ins P_3, such as those described in this chapter, it is important that the peak levels of Ins P_3 are reproducibly measured using assay conditions in which Ins P_3 metabolism is

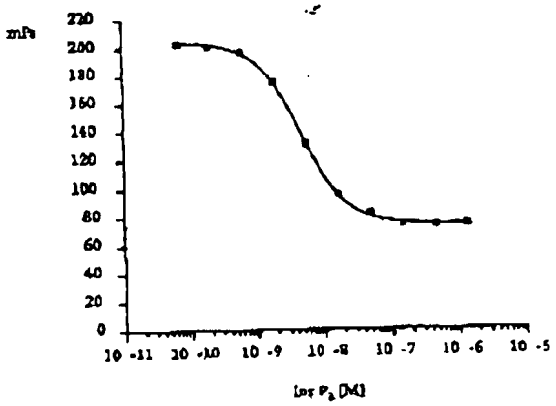


FIGURE 20.7 Automation of the HitHunter Ins P_3 FP assay. Dispensation of the reaction was done on a BioMek 2000; ten replicates for each standard concentration were run. The $IC_{50} = 5$ n*M*, the mean %CV was 2 and the Z′ factor was 0.90.

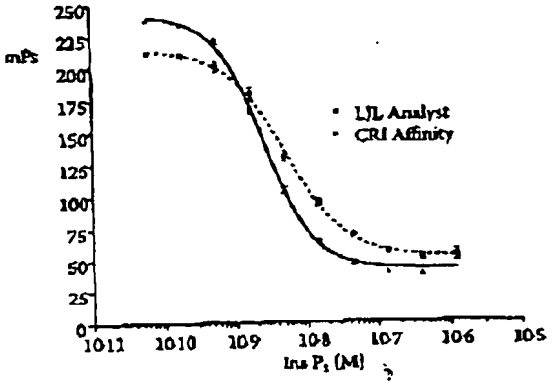
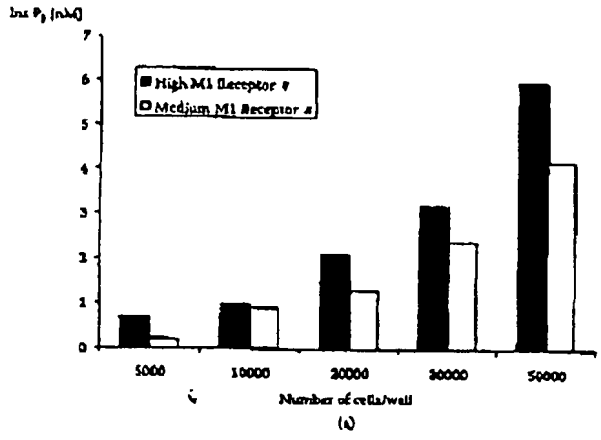


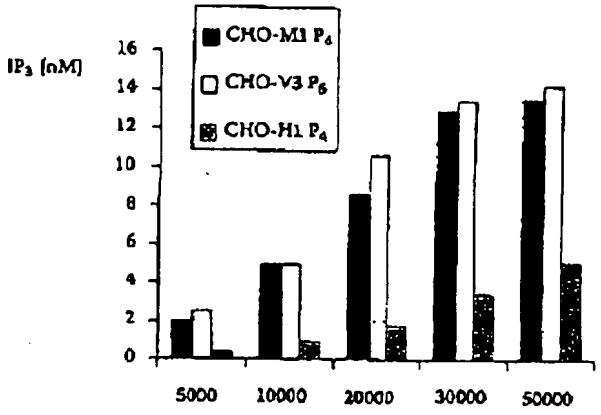
FIGURE 20.8 Comparison of two FP readers measuring the Ins P_3 FP assay. The Ins P_3 FP green tracer standard curve was run on an LJL Analyst GT and a Beckman-Coulter CRI Affinity. The LJL Analyst was set as follows: integration time = 100,000 μsec, G Factor 1.0. The filter set used in the CRI Affinity was excitation filter — fluorescein 485 nm, Emission filter — fluorescein 530 nm, and dichroic — fluorescein 50[illegible] nm. The exposure was set at 15 to 30 msec, and the focus was set at 2700 to 3200. In this particular experiment the LJL Analyst run had an $IC_{50} = 5$ n*M*, with 5% mean CV and a Z′ factor = 0.87. For the CRI Affinity, $IC_{50} = 3$ n*M*, with 3% CV and Z′ factor = 0.97.

arrested. To achieve this, the cell samples are rapidly deproteinized after agonist addition with perchloric acid (PCA; 0.2 *N*), which displaces Ins P_3 from the salts by acting as a chaotropic agent and terminates metabolic activity.

An important feature of using the assay in high-throughput robotic fluid dispensing systems is that the PCA needs to be added 20 to 30 sec after addition of the agonists, in order to measure the peak formation of Ins P_3. CHO-M1 cells induced with carbachol exhibited maximal Ins P_3 induction within 30 sec, followed by a rapid decline over the following 5 min (Figure 20.10). Similar results are seen in histamine H_1 receptor cells (Figure 20.10).



(a)



(b)

FIGURE 20.9 (a) Basal cellular Ins P_3 levels increase with cell number. Between 5000 and 50,000 CHO-K1 muscarinic M1 receptor cells (expressing either 1.5 or 8.3 pmol/mg protein of receptor) were assayed in triplicate to determine the basal amounts of Ins P_3 in the cell. No agonist was added to the cells in this experiment. Samples were assayed following the protocol shown in Figure 20.2. The amount of Ins P_3 was calculated from the standard curve run in parallel with the test conditions (data not shown). (b) Basal Ins P_3 levels in CHO-K1 cells expressing different G_q-coupled receptors. Between 5000 and 50,000 cells were assayed to measure the levels of Ins P_3 expressed by the cell lines in the absence of agonist addition. Samples were assayed in triplicate. The passage number of each cell line was noted (P_4 or P_6), as the age of the cell line can affect the expression levels of Ins P_3. The levels of detected Ins P_3 were calculated off a standard curve run in parallel with the experiment.

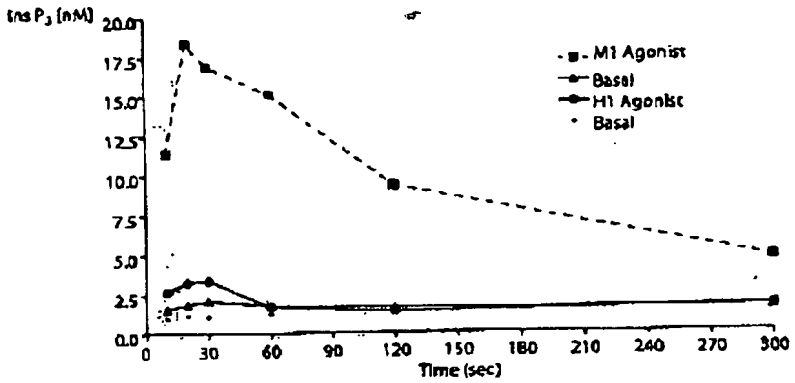


FIGURE 20.10 Monitoring agonist stimulated Ins P_3 levels in CHO-M1 cells and CHO-H1 cells over time. Twenty thousand CHO-M1 cells were treated with 1000 μM carbachol, and CHO-H1 cells were treated with 100 μM histamine. At the end of each noted time point, 0.2 N PCA was added to quench the reaction and the Ins P_3 FP assay was carried out as described above. The amount of Ins P_3 detected in the cells after the defined agonist stimulation period was calculated off an Ins P_3 standard curve run in parallel to the test samples. Samples were assayed in triplicate.

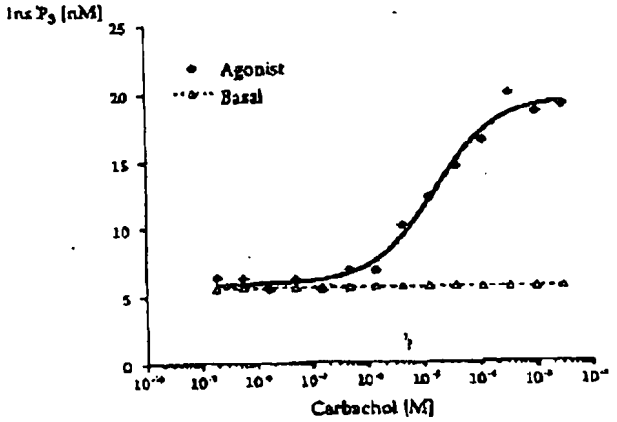


FIGURE 20.11 Agonist stimulation of CHO-M1 cells. Twenty thousand stably expressing CHO-M1 cells were treated with an increasing concentration of carbachol for 20 sec. PCA was immediately added after the agonist incubation period added to quench the reaction. The levels of Ins P_3 were extrapolated from a standard curve that was run in parallel (data not shown). The samples were assayed in triplicate. The IC_{50} of carbachol was determined to be 15 μM.

Agonist concentration response curves can be established using this assay with high potency. A prototypical receptor that induces formation of Ins P_3 is the muscarinic M_1 receptor. The agonist carbachol increased Ins P_3 levels approximately fourfold (Figure 20.11) with a potency (EC50) of 7 μM [13]. The induction of Ins P_3 was antagonized by the muscarinic antagonist atropine with potencies in a range consistent with the literature (0.1 to 10 μM) (Figure 20.12) [21], using both assay systems to detect Ins P_3. Similar data can be seen using a more potent agonist in a different receptor system (Figure 20.13), in which both full and partial agonists can be detected. Agonist responses were antagonized by compounds in a concentration-dependent fashion (Figure 20.

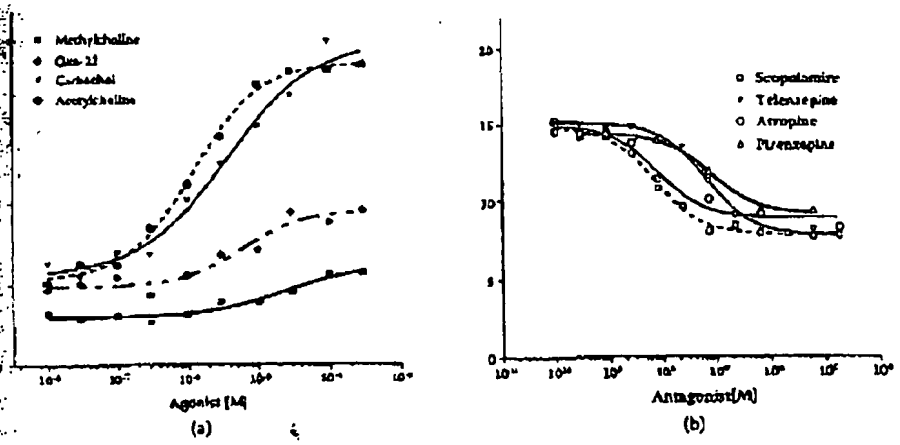


FIGURE 20.12 Agonism and antagonism of CHO-M1 receptor: (a) twenty thousand CHO- M1 cells were treated with increasing concentrations of the following known M1-specific agonists: methacholine (closed square, solid line), OX-22 (cis-2-methyl-5-trimethylammoniummethyl-1,3-oxathiolane iodide) (closed diamond, dash line), carbachol (inverted closed triangle, solid line), and acetylcholine (closed circle). (b) twenty thousand CHO-M1 cells were pretreated with increasing concentrations of the following muscarinic receptor antagonists: scopolamine hydrobromide (open square, dash line), telenzepine (inverted open triangle, solid line), atropine (open circle, solid line), and pirenzepine (open triangle, solid line) for 30 min. The antagonists were washed from the cells, and then 300 μM carbachol (previously determined EC_{80} concentration) was added to the cells in fresh medium. The carbachol induction lasted for 20 sec, and the reaction was quenched by the addition of 0.2 *N* PCA. The levels of Ins P_3 detected by agonist and antagonist treatment were extrapolated from a standard curve run in parallel with the experiment. Both analyses were performed using the Ins P_3 green tracer. (From Eglen, RM. *Combin Chem & HTS*, 2005; 8:311–318. With permission.)

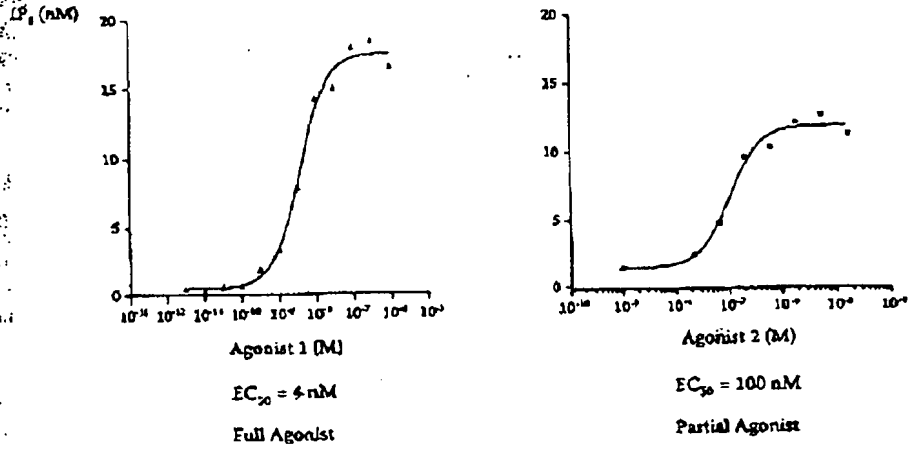


FIGURE 20.13 Concentration effect curves for Ins P_3 measurements of two antagonists at the same G-protein–coupled receptor. The agonist used to elicit the response was the full agonist shown in Figure 20.14.

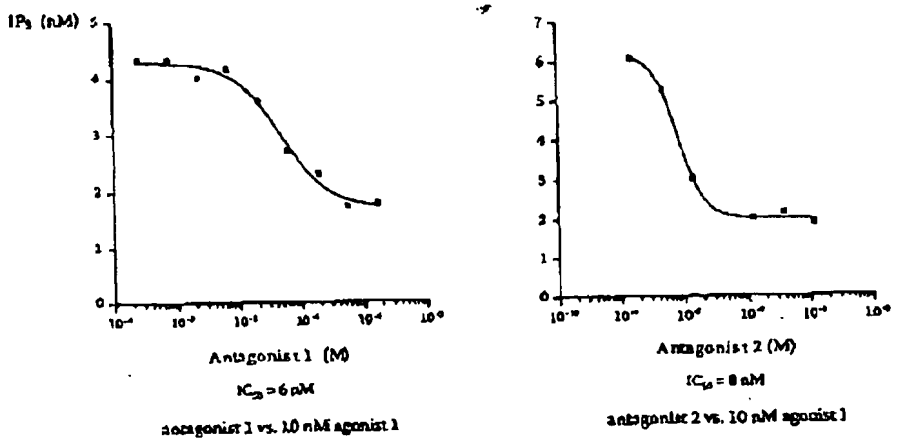


FIGURE 20.14 Concentration effect curve for Ins P_3 measurements at a Type II G-protein–coupled receptor. The agonist under investigation in the left panel is a full agonist with a high potency. The agonist under investigation in the right panel is a partial agonist.

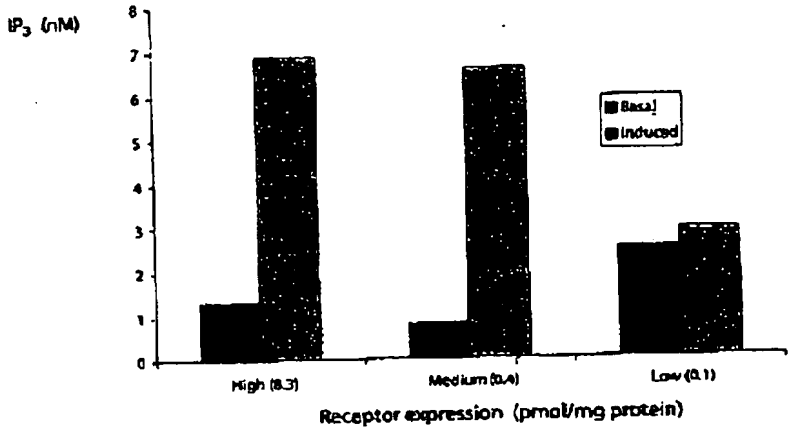


FIGURE 20.15 Correlation of receptor expression levels and detection of Ins P_3 levels in three different CHO-K1 cell lines expressing a muscarinic M_1 receptor. Three different stably transfected CHO-K1 cell lines were tested as they expressed different muscarinic M1 receptor levels [high = 8.3 pmol/mg-protein, medium = 0.4 pmol/mg-protein, and low = 0.1 pmol/mg-protein]. Twenty thousand cells per well were plated in two sets of triplicate wells. The samples were treated with either buffer (Basal) or 300 μM carbachol (induced). Shown are the results with the Ins P_3 green tracer. The concentration of Ins P_3 detected in the assay was determined from extrapolation from a standard curve run in parallel to the experimental conditions.

The low efficacy of some agonists at inducing Ins P_3 is due to the low receptor reserve associated with the response. Indeed, the response is much less well coupled to receptor activation than calcium is (see below). Consequently, it is anticipated that the maximal level of induction would be sensitive to the receptor expression levels in the cell line. This is indeed the case with CHO-M1 cells, as shown in Figure 20.15, where receptor expression levels of 0.4 pmol per mg protein and above are required.

20.6 COMPARISON OF AGONIST INDUCTION OF INS P_3 IN COMPARISON TO INTRACELLULAR CALCIUM

G_q-coupled GPCR stimulation ultimately causes the liberation of calcium from bound intracellular stores. When measuring a calcium response that is significantly downstream from the receptor, the GPCR response is highly amplified, resulting in potent agonist responses. As described above, the fluorescence imaging plate reader (FLIPR) is frequently used to measure calcium changes in living cells by means of calcium-specific fluorescent dyes (Figure 20.16). Comparison of several muscarinic agonists in assays measuring either Ins P_3 or calcium changes shows clearly marked differences in compound potencies (Figure 20.17). However, when equiactive agonist concentrations (such as the EC_{80} concentration) are used to determine antagonist potency, similar values can be found (Figure 20.16). Thus, the values for a series of muscarinic potencies (IC_{50}) determined in an Ins P_3 assay compare well with values from a FLIPR experiment. A final point is that the rapid kinetics of either calcium release or changes in Ins P_3 does not allow sufficient time for the agonist to reach equilibrium with a preincubated antagonist, resulting in a state of hemiequilibrium in which the receptors are effectively bound irreversibly during the assay period. This is most noticeable using compounds of high affinity; therefore, depression in the agonist concentration response curve maxima will be observed in either assay. FLIPR analysis can be prone to compound interferences that modulate calcium levels resulting in false negatives or positives. It is anticipated that interferences of this nature would be much less with an Ins P_3 assay. Studies have confirmed that several calcium channel blockers interfere in the FLIPR assay, including verapamil, nifedipine, nimodipine, and nitrendipine. However, they did not influence the Ins P_3 stimulation, and were not therefore false negatives in this assay.

20.7 CONCLUSIONS

Measuring GPCR activation upon ligand addition via monitoring second messenger response is a commonly used technique in screening. In screening for ligands at G_q-coupled receptors, several methods have been developed to detect agonist induced changes in Ins P_3, PI, PIP_2, PLC, and calcium. These methodologies include both homogeneous and heterogeneous formats. The HitHunter FP Ins P_3 assay is a homogeneous assay that is a sensitive, nonisotopic high-throughput assay to measure Ins P_3. This assay is highly automatable and can be used with several cell lines expressing differing levels of GPCRs. The flexibility in the assay format provides for optimizing the sensitivity of the analysis for automation and miniaturization. The variety of tracers available for the assay may also reduce library compound interference.

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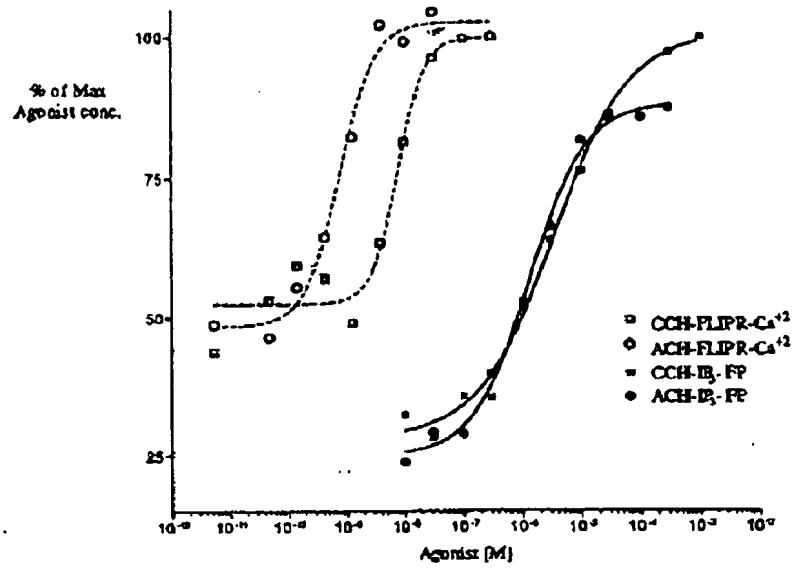

FIGURE 20.16 Agonist stimulation measured by HitHunter Ins P_3 FP and FLIPR analysis. For the FLIPR analysis (dashed line), CHO-M1 cells were plated at a density of 50,000 cells per well, while 20,000 cells per well were used in the Ins P_3 FP assay (solid line). The agonists carbachol (open or closed square) or acetylcholine (open or closed circle) were added to the cells for 20 sec, after which they cells were processed according to described protocols to measure changes in either calcium or Ins P_3 levels. For both analyses, all samples were assayed in triplicate. For FLIPR analysis, the average was taken for the peak fluorescent reading at each treatment while for Ins P_3 FP analysis, the mean FP values were extrapolated from a standard curve to determine the amount of Ins P_3. The results for both assays were normalized to the maximal agonist response.

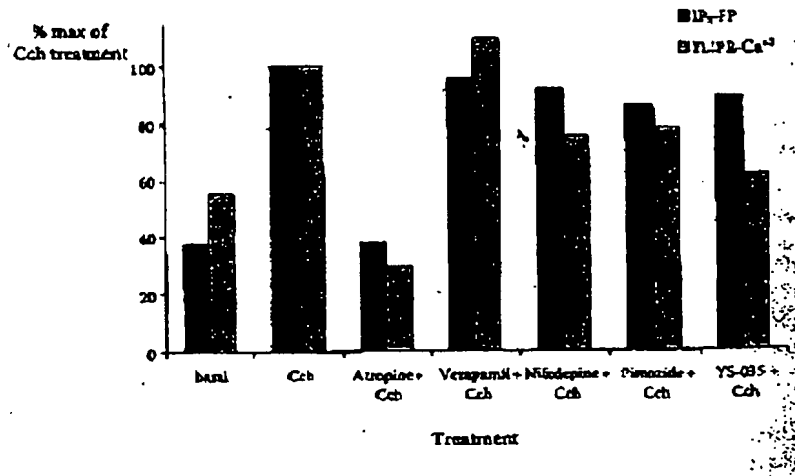

FIGURE 20.17 Effect of calcium channel blockers on the Ins P_3 FP assay. Four calcium channel blockers were examined in the HitHunter Ins P_3 FP assay. The number of cells used were 50,000 CHO-M1 cells (FLIPR analysis) or 20,000 CHO-M1 cells. As a control, carbachol was added to the cells at a concentration of 1 μM, and atropine was used a control antagonist at a concentration of 1 μM. Samples are assayed in triplicate. The different treatments are plotted against the percentage of the carbachol-alone treatment.

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